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Komari, Plant Cell Reports, vol 9, pages 303-306, 1990.

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Plant Cell Reports (1990) 9: 303–306

Plant Cell Reports

Transformation of cultured cells of *Chenopodium quinoa* by binary vectors that carry a fragment of DNA from the virulence region of pTiBo542

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Received March 30, 1990/Revised version received June 25, 1990 – Communicated by A. R. Gould

Summary. A 15.2-kb KpnI fragment from the virulence region of pTiBo542, the Ti plasmid harbored by *Agrobacterium tumefaciens* strain A281, was introduced into binary vectors. The fragment contained the *virB*, *virC* and *virG* genes, and it is known to have the ability to increase the virulence of strains of *A. tumefaciens*. The strains of *A. tumefaciens* that carried the resulting plasmids were able to transform cells in a suspension culture of *Chenopodium quinoa* Willd cells which were not transformable by common vectors. Although the sizes of the plasmids was very large, a foreign segment of DNA was introduced into one of the plasmids by homologous recombination in *A. tumefaciens* cells, and the segment was subsequently transferred to plant cells.

Abbreviations. NPT: neomycin phosphotransferase, SPT: streptomycin/spectinomycin phosphotransferase

Introduction

Agrobacterium tumefaciens is a soil bacterium that can genetically transform plant cells with a segment of DNA (transfer DNA, abbreviated as T-DNA) from a tumor-inducing plasmid (Ti plasmid) with the resultant production of a crown gall, which is a plant tumor (Bevan and Chilton 1982; Depicker et al. 1983; Nester et al. 1984). A number of sophisticated plant-transformation vectors, based on this naturally occurring gene-transfer mechanism, have been developed and such vectors are widely employed in plant molecular biology and genetic engineering (Fraley et al. 1986).

The genes responsible for the transfer of T-DNA are clustered together in another region, called the virulence region, of the Ti plasmid (Klee et al. 1983). The transfer process is active even when the virulence genes and the T-DNA are located on separate replicons in an *A. tumefaciens* cell (Hoekema et al. 1983). In a widely used binary system, one Ti plasmid serves as a helper, providing the virulence functions, and an artificial "T-DNA", which contains a selectable marker and genes of interest, is placed on a second, small plasmid, which is referred to as a binary vector (An et al. 1988).

Chenopodium quinoa Willd is a plant that is widely used in research on plant viruses, and it is also known as an important food crop in South America (Vietmeyer 1986). Transformation is an indispensable technique for molecular analysis of any plant species. We have been trying to establish a method for the transformation of *C. quinoa*, but our efforts using various strains of *A. tumefaciens* and a common binary vector have not been successful to date.

A. tumefaciens strain A281 (Watson et al. 1975) is a so-called "super-virulent" strain, and its host range is wider and its transformation efficiency is higher than those of other strains (Hood et al. 1987; Komari 1989). These characteristics are associated with the Ti plasmid, pTiBo542, harbored by this strain (Hood et al. 1984; Komari et al. 1986). The genes responsible for the "super-virulence" have been mapped to the virulence region of pTiBo542, and the virulence of A281 can be increased even further when a fragment of DNA from the virulence region is added (Jin et al. 1987).

This report describes the successful transformation of cells in a suspension culture of *C. quinoa* cells by a combination of binary vectors and the fragment from the virulence region of pTiBo542.

Materials and methods

Cell cultures. A callus culture was initiated from a segment of a leaf of a *C. quinoa* plant grown under sterile conditions. The medium for the initiation and maintenance of the culture consisted of Linsmaier and Skoog (1965) salts, 30 g/l sucrose, 3 mg/l indole-3-acetic acid, 3 mg/l naphthaleneacetic acid, 0.1 mg/l kinetin, and 0.9% agar. A suspension culture was initiated from the callus culture by using the same medium

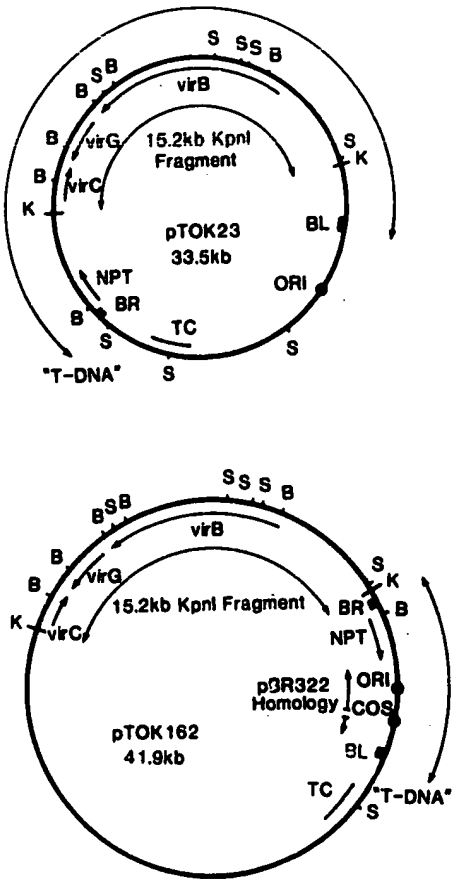

Fig. 1. Structure of pTOK23 and pTOK162. The 15.2-kb KpnI fragment from the virulence region of pTiBo542 was inserted into the KpnI sites of pGA437 (pTOK23) and pTOK154 (pTOK162). The regions to be transferred to plants are indicated as "T-DNA". The region homologous to pBR322 is shown in pTOK162. Abbreviations: BR, right border; BL, left border; TC, tetracycline-resistance gene; ORI, origin of replication of ColE1; COS, cos site of lambda phage; B, BamHI; K, KpnI; S, SalI.

without agar. The intervals for subculturing were three weeks for the callus culture and one week for the suspension culture.

Plasmids and bacterial strains. *A. tumefaciens* strains A208 (Montoya et al. 1977), A281 (Watson et al. 1975), A348 (Garfinkel et al. 1981) and LBA4404 (Hoekema et al. 1983), and plasmids pGA437, pGA472 (An et al. 1985), and pTOK47 (Jin et al. 1987) have been described previously. pTOK23 (Fig. 1) was constructed by combining a 15.2 kb KpnI fragment from the virulence region of pTiBo542 with pGA437 digested with KpnI. pTOK154 was constructed as follows. pVCK101 (Knauf and Nester 1982) was digested with EcoRI, treated with Klenow fragment of DNA polymerase I, and re-circularized. pGA472 was digested with HindIII and BglII, treated with Klenow fragment, and re-circularized. The resulting plasmid was cut with SalI, and treated with Klenow fragment. The blunt-ended SalI fragment, containing the "T-DNA", and a synthetic poly-linker, which included HindIII, SalI, XbaI, BamHI, SmaI, KpnI and SstI sites, were combined and circularized. This molecule was digested with HindIII and SalI, and then it was introduced between the HindIII and XhoI sites of the above mentioned derivative of pVCK101, to generate pTOK154. Thus, pTOK154 had SstI, KpnI, XbaI, BglII and HindIII sites as unique sites outside the "T-DNA". The 15.2-kb KpnI fragment was inserted into the KpnI site of pTOK154 to generate pTOK162 (Fig. 1). The SPT gene derived from Tn7 (DeGreve et al. 1981) was obtained as a ClaI fragment from a pBR322 clone, treated with Klenow fragment, and inserted into the SmaI site in the first intron of the fibroin gene from *Bombyx mori* (Ohshima and Suzuki 1977; Tsujimoto and Suzuki 1979) in pOTS2910 (obtained from Dr. Suzuki, National Institute for Basic Biology, Okazaki, Japan), to generate pTOK171.

Procedures for manipulation of DNA and *Escherichia coli* (strain TB1) were those described by Maniatis et al. (1982). Plasmids were introduced to *A. tumefaciens* by triparental matings (Ditta et al. 1980) or by the freeze-thaw method (An et al. 1988). *A. tumefaciens* was grown on AB medium (Chilton et al. 1974) at 28°C.

Transformation and analysis of transformants. The procedures for transformation of cultured cells have been described previously (An 1985; Komari 1989). The duration of the co-cultivation of plant cells and bacteria was 48 h. The callus-maintenance medium, supplemented with 250 mg/l cefotaxime and 300 mg/l kanamycin, was used as the selection medium. Leaf-disk transformation, isolation of DNA from plants, and Southern hybridization were performed by the methods described by Komari et al. (1989). The probes used were the 2.1-kb BamHI-HindIII fragment from pGA472 (NPT probe) and the 1.4-kb HindIII-XhoI fragment from pOTS2910.

Results

Initially, I tried to transform *C. quinoa* by using pGA472, a common binary vector, in combination with four strains of *A. tumefaciens*, namely, A208, A281, A348, and LBA4404. Leaf disks, a callus culture, and a suspension culture of *C. quinoa* were used in various experiments, but all of the trials failed (Table I). Then we considered using the 15.2-kb KpnI fragment from the virulence region of pTiBo542. This fragment contains the *virB*, *virC* and *virG* genes. It has been reported that A281, when it carried a plasmid with a wide host range, that contained the KpnI fragment, was more virulent than A281 by itself (Jin et al. 1987). We had previously constructed pTOK47 by combining the KpnI fragment and a plasmid with the origin of replication of pSa, which was compatible with various derivatives of pRK2. Although pGA472 is a derivative of pRK2, a strain that stably carried both pTOK47 and pGA472 could not be obtained for unknown reasons. Therefore, I decided to introduce the KpnI fragment to a binary vector.

The prototype plasmid that I constructed was designated pTOK23 (Fig. 1). pTOK23 was obtained by introducing the KpnI fragment into pGA437, another binary vector. Cells from a suspension culture of *C. quinoa* were co-cultivated with A281(pTOK23) and selected for resistance to kanamycin, and kanamycin-resistant colonies were obtained (Fig. 2, Table I). Thus, the combination of a binary vector and the KpnI

Table I. Transformation of cells in a suspension culture of *C. quinoa* cells. Numbers of kanamycin-resistant calli appearing per 60-mm petri dish are shown. Approximately 10^4 cell clusters from the suspension culture were plated on a petri dish. Each cluster consisted of 10 - 30 cells. Results are the averages of five independent experiments with standard deviations.

Plasmid	Strain			
	LBA4404	A281	A348	A208
pGA472	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
pGA437	0.0 ± 0.0	0.0 ± 0.0		
pTOK23	0.0 ± 0.0	9.4 ± 4.4		
pTOK154	0.0 ± 0.0	0.2 ± 0.4		
pTOK162	3.4 ± 1.1	10.8 ± 5.0		

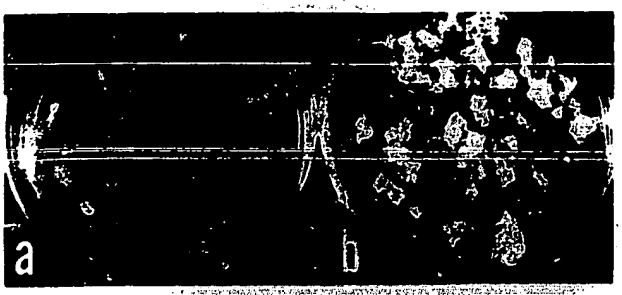

Fig. 2. Kanamycin-resistant calli as they appeared after co-cultivation. The suspension culture of *C. quinoa* was co-cultivated with (a) A281(pGA472) or (b) A281(pTOK23) for 48 h, and plated on kanamycin-containing medium. The photograph was taken after growth for 6 weeks on this medium.

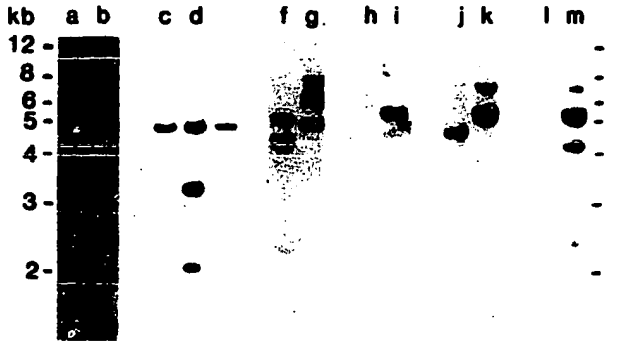

Fig. 3. Southern hybridization. DNA was isolated from plant cells, digested with restriction enzymes, subjected to electrophoresis on agarose gels, transferred to nylon membranes, and probed with ^{32}P-labeled DNA fragments. Lanes a and b, BamHI digests of DNA from *C. quinoa*, which was either not transformed (a) or transformed (b) with A281(pTOK23), hybridized to the NPT probe; lanes c through e, BamHI-EcoRI digests of DNA from *C. quinoa* transformed with LBA4404(pTOK162) (c), A281(pTOK162) (d) and A281(pTOK154) (e), hybridized in each case to the NPT probe; lanes f and g, HindIII digests of DNA from *C. quinoa* transformed with A281(pTOK162::pTOK171) hybridized to the NPT probe; lanes h and i, material in lanes f and g was re-hybridized to the probe specific for pTOK171; lanes j and k, HindIII digests of DNA from tobacco plants transformed with LBA4404(pTOK162::pTOK171) hybridized to the NPT probe; lanes l and m, material in lanes j and k was re-hybridized to the probe specific for pTOK171.

fragment made possible the transformation of *C. quinoa*.

The structure of pTOK23 may be unusual, perhaps because the virulence genes were in the "T-region", and further manipulations appeared to be limited. An additional molecular construction was generated. A binary vector, pTOK154, was constructed from pGA472 and pVCK101, a plasmid with a wide host range. pTOK154 had several restriction sites, which included a KpnI site outside the "T-region". Various virulence fragments can be introduced into those sites and tested. The KpnI fragment was introduced into the KpnI site of pTOK154 to generate pTOK162 (Fig. 1).

A281(pTOK162) was able to transform cells in the suspension culture of *C. quinoa* cells quite efficiently (Table I). In addition, LBA4404(pTOK162) was able to do so at a somewhat lower frequency. Since LBA4404(pTOK23) was unable to transform the cultured cells, pTOK162 was deemed superior to pTOK23. It is noteworthy that A281(pTOK154) transformed the cultured cells at a very low frequency; only one transformed callus was obtained throughout the course of the experiments. The superiority of pTOK162 to pTOK23 seemed to reflect the properties of the precursor plasmids, pTOK154 and pGA437, although the reason for the difference is not clear.

Both pTOK23 and pTOK162 were huge plasmids with numerous restriction sites. It is not very easy to introduce genes of interests into the "T-region" of pTOK162 by standard sub-cloning techniques. One possible technique for this purpose involves the use of homologous recombination in an *A. tumefaciens* cell (Herrera-Estrella et al. 1983; Horsch et al. 1984). pTOK162 has DNA sequences that are homologous to those of pBR322. If a derivative of pBR322 is introduced into an *A. tumefaciens* cell that carries pTOK162, a single, homologous recombination can cause the derivative of pBR322 to become integrated into pTOK162. Since pBR322 cannot replicate in *A. tumefaciens* cells, such recombination represents the only way for the derivative of pBR322 to survive in *A. tumefaciens* cells. Any *A. tumefaciens* cells that carries a recombinant, namely the pTOK162::pBR322 derivative, has the drug-resistance markers of both plasmids and should be readily selectable.

The potential utility of this method was tested. Various plasmids were introduced into A281(pTOK162) and LBA4404(pTOK162). I found that the spectinomycin-resistance gene (SPT) derived from Tn7 was an efficient selection marker for the derivatives of pBR322. An example of the plasmids is provided by pTOK171, which consisted of the SPT gene and an insect-derived gene on pBR322. pTOK171 was introduced into A281(pTOK162) and LBA4404(pTOK162) by triparental matings. By selecting for resistance to kanamycin, the marker for pTOK162, and resistance to spectinomycin, the marker for pTOK171, I was able easily to obtain *A. tumefaciens* cells that might contain the recombinant plasmids. Restriction analyses of "mini-preparations" of the plasmids from [illegible] cells confirmed the expected structures (data not shown). A281 cells that carried pTOK162::pTOK171 were able to produce kanamycin-resistant transformants of *C. quinoa*. In addition, tobacco leaf disks were transformed with LBA4404(pTOK162::pTOK171), and several kanamycin-resistant, regenerated plants were obtained.

The DNA isolated from selective transformants was analyzed by Southern hybridization (Fig. 3). At least one fragment of DNA homologous to the NPT probe was identified in each f the kanamycin-resistant transformants of *C. quinoa* analyzed (lane b - g). The probes specific for pTOK171 was also used to analyze the DNA from seven transformants pr duced by A281(pTOK162::pTOK171). The expected hybridization band (5.5-kb HindIII fragment) was

detected in four samples (an example in lane i), but they were absent fr m the other transformants analyzed (an example in lane h), possibly as a result of rearrangements of DNA during the transformation processes. When the DNA from two tobacco plants transformed with LBA4404(pTOK162::pTOK171) was analyzed, the gene for NPT was detected in both samples (lane j and k), and the sequence derived from pTOK171 was found in one of the plants (lane m).

Discussion

A limited number of plant species, including tobacco, have been heavily favored in plant transformation experiments. The techniques developed for these species may not be readily applicable to other species of plants. We faced such a problem when we tried to transform *C. quinoa*. The present study exploited the fragment of DNA that is responsible for the "super-virulence" phenotype of a strain of *A. tumefaciens*, in order to overcome the problem. The superior ability of the binary vectors that carried the KpnI fragment from the virulence region of pTiBo542 in the transformation of *C. quinoa* was evident. Although rearrangements of the transferred segments of DNA from pTOK162 and its derivatives were observed in some cases, such rearrangements are not uncommon in other transformation systems. These data imply that the utilization of the KpnI fragment may also be helpful in the transformation of other plant species.

One of the plasmids, pTOK162, was shown to have the ability to accept various sequences of DNA and to transfer them to *C. quinoa*. If a pBR322 clone of a gene of interest is available, such as pOTS2910, the gene can be introduced into pTOK162 by homologous recombination in *A. tumefaciens* cells, after the SPT gene has been inserted to the derivative of pBR322. Alternatively, a gene of interest can be introduced into a plasmid that consists of the pBR322-derived sequence and the SPT gene.

The system provided by pTOK162 was efficient, but it depends on complicated processes, for example, homologous recombination in *A. tumefaciens*. This system was primarily designed to test various virulence fragments. Since the KpnI fragment was proven to increase the efficiency of binary systems in this study, construction of plasmids similar to pTOK47 through the use of plasmids that belong to other incompatibility groups offers reasonable approach to this problem for future study. Nevertheless, the successful transformation of *C. quinoa* described herein represents a very important step towards molecular studies of this plant species.

Extensive efforts towards the regeneration of plants from cultured cells or protoplasts of *C. quinoa* have been made, but have been unsuccessful (Kumashiro, personal communication). Further improvements in techniques of tissue culture for *C. quinoa* are needed, before we can generate transgenic plants.

LBA4404(pTOK162::pTOK171) was able to generate a transgenic tobacco plant which carried the pTOK171-derived sequence of DNA. This result provides an indication that the pTOK162 system will be applicable to various plant species.

Acknowledgements. The author thanks Dr. G. An of Washington State University for the gift of pGA437 and pGA472, Dr. Y. Suzuki of National Institute for Basic Biology, Okazaki, Japan for the gift of pOTS2910, Y. Saito for technical assistance, and T. Kumashiro for valuable advice.

References

An G (1985) Plant Physiol 79:568-570
An G, Evert PR, Mitra A, Ha SB (1988) In: Gelvin SB, Schilperoort RA (ed) Plant Molecular Biology Manual A3. Kluwer Academic, Dordrecht, pp 1-19
An G, Watson BD, Stachel S, Gordon MP, Nester EW (1985) EMBO J 4:277-284
Bevan MW, Chilton M-D (1982) Annu Rev Genet 16:357-384
Chilton M-D, Currier TC, Farrand SK, Bendich AJ, Gordon MP, Nester EW (1974) Proc Natl Acad Sci USA 71:3672-3676
DeGreve H, Decraemer H, Seurinck J, Van Montagu M, Schell J (1981) Plasmid 6:235-248
Depicker A, Van Montagu M, Schell J (1983) In: Kosuge T, Meridith CP, Hollaender A (ed) Genetic Engineering of Plants: an Agricultural Perspective. Plenum Publishing Corp, New York, pp.143-176
Ditta G, Stanfield S, Corbin D, Helinski DR (1980) Proc Natl Acad Sci USA 77:7347-7351
Fraley RT, Rogers SG, Horsch RB (1986) CRC Crit Rev Plant Sci 4:1-46
Garfinkel DJ, Simpson RB, Ream LW, White FF, Gordon MP, Nester EW (1981) Cell 27:143-153
Herrera-Estrella L, Depicker A, Van Montagu M, Schell J (1983) Nature 303:209-213
Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) Nature 303:179-180
Hood EE, Fraley RT, Chilton M-D (1987) Plant Physiol 83:529-534
Hood EE, Jen G, Kayes L, Kramer J, Fraley RT, Chilton M-D (1984) Bio/Technology 2:702-709
Horsch RB, Fraley RT, Rogers SG, Sanders PR, Lloyd A, Hoffmann N (1984) Science 223:496-498
Jin S, Komari T, Gordon MP, Nester EW (1987) J Bacteriol 169:4417-4425
Klee HJ, White FF, Iyer VN, Gordon MP, Nester EW (1983) J Bacteriol 153:878-883
Knauf V, Nester E (1982) Plasmid 8:45-54
Komari T (1989) Plant Science 60:223-229
Komari T, Halperin W, Nester EW (1986) J Bacteriol 166:88-94
Komari T, Saito Y, Nakakido F, Kumashiro T (1989) Theor Appl Genet 77:547-552
Linsmaier EM, Skoog F (1965) Physiol Plant 18:100-127
Maniatis T, Fritsch EF, Sambrook J (1982) Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
Montoya AL, Chilton M-D, Gordon MP, Sciaky D, Nester EW (1977) J Bacteriol 129:101-107
Nester EW, Gordon MP, Amasino RM, Yanofsky MF (1984) Annu Rev Plant Physiol 35:387-413
Ohshima Y, Suzuki Y (1977) Proc Natl Acad Sci USA 74:5363-5367
Tsujimoto Y, Suzuki Y (1979) Cell 16:425-436
Vietmeyer ND (1986) Science 232:1379-1384
Watson B, Currier TC, Gordon MP, Chilton M-D, Nester EW (1975) J Bacteriol 123:255-264.